

# OSTEOARTHRITIS and CARTILAGE

## The effect of strenuous versus moderate exercise on the metabolism of proteoglycans in articular cartilage from different weight-bearing regions of the equine third carpal bone

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### Summary

Articular cartilage degeneration in the middle carpal joint is a common problem in racing horses. This study evaluated the effect of exercise on the *in-vitro* synthesis of the large aggregating proteoglycans (aggrecan) and two small proteoglycans, biglycan and decorin, in articular cartilage taken from three weight bearing regions of the third carpal bone of horses which were subjected to moderate or strenuous exercise. Twelve Standardbred horses free from clinical and radiographic disease of the middle carpal joint were subjected to an 8 week moderate exercise program. The horses were then randomly assigned to two groups: group A—continued moderate exercise and group B—strenuous exercise for 17 weeks. Horses were then rested for 16 weeks. Full-depth articular cartilage explants from the dorsal radial facet (DRF), dorsal intermediate facet (DIF) and palmar condyle (PC) of the third carpal bone were collected and cultured. Cartilage proteoglycan content and release into culture media were measured. Newly synthesized proteoglycans were labeled with  $^{35}\text{SO}_4^{2-}$  for 48 h and analyzed by size exclusion and hydrophobic chromatography, sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) and autoradiography. Histologic sections of adjacent osteochondral regions were evaluated for evidence of arthritic change. No histologic abnormalities or differences in proteoglycan content were detected in any of the articular cartilage regions examined. There was however, a significant reduction ( $P < 0.05$ ) in aggrecan synthesis and a concomitant increase in decorin synthesis ( $P < 0.05$ ) in articular cartilage from the DRF of group B animals. There was no change in biglycan synthesis, aggrecan hydrodynamic size or ability to aggregate in any articular cartilage region. This study has demonstrated that strenuous exercise in horses can lead to a disturbance in the biosynthesis of proteoglycans in articular cartilage regions subjected to high contact stresses (DRF). These metabolic abnormalities, which persisted for 16 weeks after cessation of exercise, could have deleterious effects on the biomechanical properties of the tissue. We suggest that the observed alteration in articular cartilage metabolism in CRF cartilage of strenuously exercised horses could represent a predisposing factor for cartilage degeneration and osteoarthritis at a later stage.

Key words: Proteoglycans, Metabolism, Exercise, Equine, Carpal.

### Introduction

TRAUMATIC arthritis of the middle carpal joint is a common problem in racing Standardbred and Thoroughbred horses [1]. Degenerative joint diseases including osteoarthritis (OA) and related chip fractures were the cause of 28% of equine lameness cases in one study [2], and 42% of the cases in another [3]. Despite the huge economic importance of joint disease and OA in horses, our

understanding of the pathophysiological mechanisms involved in joint degeneration in this species is very poor. It is apparent, however, that failure of articular cartilage represents an early and critical event in the degenerative process [4]. Once damaged, articular cartilage has a limited ability to repair and this is the major limiting factor in successful rehabilitation after joint injury in horses [4, 5].

Articular cartilage covers the ends of bones and in conjunction with synovial fluid, provides an almost frictionless weight bearing surface essential for normal joint function. The extracellular matrix of equine articular cartilage, as with other species, is composed essentially of hydrated

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proteoglycans (PGs) embedded in a network of type II collagen fibers [6, 7]. The immobilization of the hydrated large aggregating PGs ('aggrecan') within the matrix gives the tissue resilience (the ability to recover from compressive deformation) [8]. Aggrecan is the most abundant PG type in articular cartilage in terms of mass, however, two small PGs, biglycan and decorin, have been isolated from articular cartilage of other species and may be present in similar molar concentrations to aggrecan [9, 10]. The glycosaminoglycan (GAG) chains of biglycan and decorin in articular cartilage contain up to 45% iduronic rather than glucuronic acid and as such these PGs have been collectively called the dermatan sulphate PGs (DSPGs). There is *in-vitro* evidence that the functions of DSPGs in connective tissues may be to modulate cell adhesion, cell replication and collagen fibrillogenesis [11–14]. We have previously demonstrated increased synthesis of both biglycan and decorin in the early stages of an experimental model of OA in sheep [15].

OA is a disease that leads to the progressive destruction of articular cartilage accompanied by profound sub-chondral bone changes, resulting in pain, lameness and eventually loss of joint function. The factors responsible for articular cartilage degeneration in equine athletes are poorly defined. In the dog, daily long-distance running in contrast to less stressful exercise has been shown to result in loss of PGs, disruption of the surface collagen fiber organization and a decrease in the equilibrium shear modulus of the articular cartilage from high weight-bearing regions of the stifle joints [16, 17]. In horses, supra-physiological and/or repetitive mechanical stress are similarly believed to play an important role in the failure of articular cartilage [18]. In the carpus, the dorsal regions of the radial, intermediate and third carpal bones of horses are subject to intermittent high impact forces during exercise, while the palmar aspect of the bones experience continual lower level loads [18–20]. These dorsal

high weight-bearing areas of the carpal bones have a high incidence of injury and cartilage degeneration whereas the palmar regions of these bones are rarely affected [18, 21, 22].

In the present study the effect of strenuous versus moderate exercise on the articular cartilage from high and low stress regions of the third carpal bone were evaluated. The metabolism of aggrecan, biglycan and decorin in explant cultures of articular cartilage from three defined weight-bearing regions of the third carpal bones of moderately and strenuously exercised horses was determined. In conjunction, histopathological and immunohistological evaluation of the cartilage was undertaken.

## Materials and Methods

### ANIMALS AND EXERCISE PROTOCOL

Twelve Standardbred horses (all castrated males ranging from 3–5 years of age, with an average body weight of  $421 \pm 10$  kg) that had been out of training for at least 4 months underwent a graded exercise program on a high-speed treadmill. The horses were involved in a study investigating the physiological effects of overtraining and subsequent detraining (Tyler *et al.* submitted for publication). Thus, following the termination of the training protocol the horses were rested in small yards or stalls for 16 weeks prior to euthanasia. The horses were clinically and radiographically free of disease affecting the carpal joints before commencing the study. The exercise program (outlined in Table I) began with an endurance phase lasting 8 weeks (phase 1) followed by a high intensity phase of 8 weeks duration (phase 2). The horses were then randomly divided into two groups for phase 3 of the training which lasted for 17 weeks. Group A ('moderate exercise') trained with increasing intensity and duration every 4 weeks while group B ('strenuous exercise') had increases in training intensity and duration every 2 weeks. In phase 4, the horses were

Table I  
*Exercise protocol for horses*

Phase 1	3 days/week: 2000 m @ 6 m/s increasing to 4000 m @ 6 m/s	
Phase 2	4 days/week: two low intensity and two high intensity sessions Low: 3000 m @ 8 m/s increasing to 4000 m @ 8 m/s High: 100% $\text{VO}_{2\text{max}}$ for $2 \times 2$ min increasing to $3 \times 2$ min	
	Group A	Group B
Phase 3	Three low intensity and two high intensity Low: increase 500 m every 4 weeks High: increase 1 min every 4 weeks	Two low intensity and three high intensity Low: increase 500 m every 2 weeks High: increase 1 min every 2 weeks
Phase 4	Stall rest with 2000 m walking twice/week	

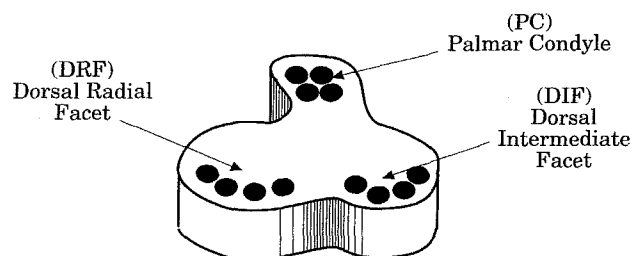


FIG. 1. Schematic representation of the equine third carpal bone showing the cartilage regions sampled for biochemical studies. Black circles represent 2 mm diameter full-thickness explants removed with a rotary trephine.

rested for 16 weeks as described earlier. All training sessions were conducted with the treadmill on a 10% slope and commenced with a warm up of 1000 m at 4 m/s.

#### CARTILAGE COLLECTION AND CULTURE

At the termination of phase 4 the horses were euthanized and the left and right carpal joints were collected and transported on ice to the laboratory. All middle carpal joints were opened under sterile conditions in a laminar-flow hood and the articular surfaces examined. Four articular cartilage explants (3 mm diameter, full thickness) were collected from the dorsal radial facet (DRF), dorsal intermediate facet (DIF) and palmar condyle (PC) of the third carpal bone as outlined in Fig. 1. The explants were cultured at 37°C in an atmosphere of 5% CO<sub>2</sub> in air with 98% humidity for 72 h in Hams F12 medium (Cytosystems, Castle Hill, NSW, Australia) supplemented with 76 mM NaHCO<sub>3</sub>, 20 mM HEPES buffer, 50 µg/ml gentamicin sulfate and 10% heat inactivated fetal bovine serum (Commonwealth Serum Laboratories, Parkville, Victoria, Australia) as previously described [15]. The media was changed every 24 h and was supplemented with 5 µCi/ml <sup>35</sup>SO<sub>4</sub>H<sub>2</sub> (Du Pont Ltd, North Ryde, NSW, Australia) for the final 48 h of culture. Media from each explant was collected and pooled separately over the 72 h culture period and stored at -20°C until analyzed.

#### PROTEOGLYCAN EXTRACTION

At the termination of culture three of the four explants from each region were pooled and extracted for 48 h at 4°C in 0.5 ml of 4.0 M guanidinium hydrochloride (GuHCl), 0.15 M sodium acetate pH 6.3 containing the proteinase inhibitors EDTA (25 mM), 6 amino-hexanoic

acid (25 mM), N-ethyl maleimide (10 mM) and benzamidine hydrochloride (5 mM).

#### CARTILAGE PROTEOGLYCAN CONTENT AND SYNTHESIS

The remaining single explant and its respective media from each region were digested separately with papain [23]. Total PG, measured as sulfated GAG, was determined in aliquots of the digest by reaction with the metachromatic dye 1,9-dimethyl-methylene blue (DMB; Sigma-Aldrich) using bovine tracheal chondroitin sulfate (Sigma-Aldrich) as a standard [24]. PG synthesis was determined by measuring incorporation of <sup>35</sup>SO<sub>4</sub><sup>2-</sup> into macromolecular PG in the papain digest after prior removal of unincorporated radiolabel using barium sulfate precipitation [24]. DNA content of cartilage explants was determined fluorometrically with Hoeschst 33258 dye (Sigma-Aldrich) using calf thymus DNA as a standard [25]. Cartilage which had been extracted with GuHCl was digested and processed as described above to determine DNA content and percentage extraction of PGs.

#### HYDROPHOBIC AND ANION EXCHANGE CHROMATOGRAPHY

To selectively concentrate the DSPGs in cartilage extracts before electrophoresis, hydrophobic chromatography on octyl-Sepharose CL4B was performed as previously described [15]. This procedure separates the cartilage extract into two pools, one which binds to octyl-Sepharose (OS-bound) and is enriched in the DSPGs and one which fails to bind to the column (OS-unbound) and contains only aggrecan. Briefly, the cartilage extracts were diluted 1:1 with 0.15 M sodium acetate pH 6.3 to give a final composition of 2 M GuHCl, 0.15 M sodium acetate pH 6.3. These samples were applied to 1 ml columns of octyl-Sepharose CL4B equilibrated in the same 2 M GuHCl/0.15 M sodium acetate buffer. The columns were developed with three volumes of starting buffer to remove the OS-unbound material, and the eluant then changed to 6 M GuHCl, 0.15 M sodium acetate pH 6.3 (three volumes) to remove the OS-bound PGs. The radioactivity in the bound and non-bound PG fractions was measured by liquid scintillation photometry.

The media samples were precipitated with three volumes of absolute ethanol and the precipitates washed twice with 75% ethanol. PGs were separated from non-glycosylated proteins by anion exchange chromatography on DEAE-Trisacryl (IBF biotechnics, France) as described previously

[15]. Briefly, the washed precipitates were redissolved in 6 M urea containing 0.15 M NaCl<sub>2</sub>, 0.02 M Tris pH 7.4 and applied to 1 ml columns of DEAE Trisacryl equilibrated in the same buffer. The columns were washed with three volumes of 0.15 M NaCl<sub>2</sub>, 0.02 M Tris and the PGs eluted with three volumes of 4 M GuHCl. The OS-bound, OS-unbound and media PG samples were then precipitated with three volumes of ethanol, collected by centrifugation, washed twice with 75% ethanol, redissolved in distilled water and freeze dried.

#### SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Aliquots of freeze dried OS-bound PGs were dissolved in sample buffer (1% SDS, 1%  $\beta$ -mercaptoethanol, 0.0006% bromophenol blue, 16% glycerine, 80 mM Tris, pH 6.8) at a concentration of 2 mg/ml and heated to 100°C for 5 min. Samples were electrophoresed at 120 V for 2 h on 1.5 mm, 15-well 4–12% gradient gels (Novex Australia, Terry Hills, Australia) containing a high concentration (0.375 M) of Tris as previously described [15, 26]. The gels were fixed in 40% methanol, 8% acetic acid and preserved by washing in 40% methanol, 5% glycerol for 1 h and dried between cellophane. Representative samples of freeze dried OS-bound PGs were also dissolved in 100 mM Tris HCl, 0.03 M sodium acetate pH 8.0 and digested with chondroitinase ABC (0.01 U/ $\mu$ g) (Sigma-Aldrich) for 12 h before electrophoresis as described above. The distribution of <sup>35</sup>S-PGs in dried SDS-PAGE gels was determined by phosphor screen autoradiography using a PhosphorImager® (Molecular Dynamics, Balwyn North, Victoria Australia) which has a linear response over a range of five orders of magnitude. The proportion of loaded <sup>35</sup>SO<sub>4</sub><sup>2-</sup> in each electrophoretic band was determined by integration of these bands using the Image Quant® software supplied with this equipment.

#### SIZE EXCLUSION CHROMATOGRAPHY

Aliquots of freeze dried PGs from OS-unbound and media samples of both group A and B horses (three regions/joint) were dissolved in 0.5 M sodium acetate, pH 6.8 and chromatographed under associative conditions in the presence of 0.1 mg/ml hyaluronan (HA) (Healon®, Pharmacia) on a Sephacryl S-1000 HR (Pharmacia) column (0.8×60 cm) equilibrated with the same buffer. OS-unbound PG samples were also chromatographed under dissociative conditions

(4 M GuHCl in the absence of added HA). The column was eluted at 20 ml/h and 1 ml fractions were collected and monitored for radioactivity and DMB positive material. The void and total volume of the column were determined with aggregated bovine nasal A1-D1 PGs and <sup>35</sup>S, respectively.

#### HISTOLOGICAL AND IMMUNOHISTOLOGICAL EXAMINATION

Full-thickness cartilage adjacent to the area of explant removal was collected from the three regions of all third carpal bones. The samples were immediately placed into separate 20 ml bottles containing 10% (v/v) neutral buffered formalin, fixed for a maximum of 24 h at room temperature and routinely processed and embedded in paraffin wax. Osteochondral slabs (approximately 5 mm in width and 5 mm deep, extending 1 cm in from the dorsal joint margin) were removed from the radial facet of horses in both groups A and B. Osteochondral samples were decalcified for 72 h in 5% (v/v) formic acid, 5% (v/v) formalin with continuous agitation and then processed as for cartilage specimens. Deparaffinized 8  $\mu$ m chondral and osteochondral sections were stained with toluidine blue O (BDH Ltd, Poole, England) and counterstained with fast green (Searle Diagnostic, High Wycombe, Bucks, England) using the method of Getzy *et al.*, [27]. Immunolocalization studies using monoclonal antibodies 3B3/C1 (ICN Pharmaceuticals (Australia), Seven Hills, NSW, Australia) and 7D4 (a generous gift from Professor Bruce Caterson, University of Cardiff, Wales) were performed in the absence of any prior enzymatic digestion essentially as described by Visco *et al.*, [28], except that a cassette and coverplate system (Shandon Lipshaw, FSE Pty. Ltd., Homebush, NSW, Australia) were used to process the slides.

#### STATISTICAL ANALYSIS

Data were normalized by square root or logarithmic transformation. The mean  $\pm$  standard error for transformed regional data from all group A joints and group B joints were compared in all analyses. A two factor analysis of variance (ANOVA) of transformed data was used to test for significant differences associated with joint region and exercise group. A Fisher's analysis of least significant difference was used to test for specific regional differences within groups. Differences between mean values for specific regions of group A and B joints were evaluated by an unpaired two

tailed Student's *t*-test. Differences were considered significant when  $P \leq 0.05$ .

## Results

### GROSS AND HISTOLOGICAL MORPHOLOGY

Small punctate (<1 mm diameter) depressions in the articular cartilage surface of the radial facet of the third carpal bone, approximately 5–10 mm from the dorsal margin of the bone, were intermittently observed in both group A and B horses. There was no difference in the apparent frequency of these lesions between the two groups. No other gross abnormalities were observed in the middle carpal joints of horses in either exercise group. Histological examination of the osteochondral sections revealed that the cartilage depressions described above appeared to be associated with collapse of the subchondral bone [Fig. 2(B)]. The cartilage sections from all regions of both groups of horses, showed no abnormalities, having normal architecture and toluidine blue staining [demonstrated in Fig. 2(A)].

Immunolocalization with antibodies 3B3(–) and 7D4 revealed mild positive staining restricted to the most superficial layer of chondrocytes in all regions of group A and B horses (Fig. 3). There was no territorial or interterritorial matrix staining with either monoclonal antibody in any of the sections from either group of horses. There was no difference between group A and B horses with regard to the degree of positive staining with these antibodies.

### CARTILAGE DNA CONTENT

The DNA content (mean standard error) of cartilage explants from the different joint regions of group A and B horses ranged from  $2.2 \pm 0.08$ – $3.4 \pm 0.43$   $\mu\text{g}$  DNA/explant. There was no significant difference in the DNA content per explant between the different joint regions of either group of horses. Furthermore there was no change in the micrograms of DNA per explant in any joint region of group A compared with group B horses. Given these findings, and the fact that cartilage wet weight has been shown to change with the onset of OA [15], all subsequent analyses were expressed on a per DNA basis.

### CARTILAGE PROTEOGLYCAN CONTENT, RELEASE INTO CULTURE MEDIA AND SYNTHESIS

There was no significant difference in the release of resident (DMB positive) PGs into the culture

media ( $\mu\text{gPG}/\mu\text{g DNA}$ ) between the three joint regions in moderately exercised (group A) horses [Fig. 4(A)]. In the overtrained animals (group B) there was an apparent increase in the release of PGs into the media in the DRF region compared with group A, however this did not reach statistical significance ( $P=0.3$ ). Nevertheless in group B horses, in contrast to group A animals, a significant difference in the release of PGs from the different joint regions was evident, with the DRF releasing significantly more PGs into the culture media than the other areas ( $P=0.001$  and  $0.02$  for DRF versus DIF and PC, respectively). There was no significant difference in the cartilage matrix PG content as measured by sulfated GAG ( $\mu\text{g}/\mu\text{g DNA}$ ), between the three joint regions in horses from either group A or B [Fig. 4(B)]. The cartilage PG content was not significantly different in any of the joint regions of group A compared with group B horses.

There was no difference in the quantity of newly synthesized PGs ( $^{35}\text{S}$ -PGs) released into the culture media or retained in the matrix (DPM/ $\mu\text{g DNA}$ ) between the three joint regions in horses from either group A or B [Figs 5(A) and 5(B), respectively]. The release of  $^{35}\text{S}$ -PGs into the culture media by all regions did not differ between group A and group B horses [Fig. 5(A)]. However, the incorporation of  $^{35}\text{S}$ -PGs into the cartilage matrix was significantly lower in the DRF region of group B compared with group A horses [Figure 5(B),  $P=0.04$ ].

### SYNTHESIS OF AGGREGAN, BIGLYCAN AND DECORIN

The  $^{35}\text{S}$ -PGs, which were extracted from the cartilage matrix of the different joint regions of the two groups of horses, were separated by hydrophobic chromatography and SDS-PAGE. The percentage of  $^{35}\text{S}$ -PGs which were extracted from the cartilage of different weight, bearing areas varied from  $57 \pm 5$  to  $65 \pm 4\%$ . There was no significant difference between regions or between Groups A and B horses with regard to this extraction percentage.

The electrophoretic distribution of representative  $^{35}\text{S}$ -PG samples is shown in Fig. 6. The OS-bound PGs from all regions of group A and B horses separated into three broad but distinct bands on SDS-PAGE [Fig. 6(A), lanes 1 and 2]. The first band, which failed to enter the resolving gel was consistent in size with aggrecan monomer. The second and third bands ( $M_r \sim 230$  and  $116$  kDa) migrated with the expected distribution of biglycan and decorin, respectively [15, 26]. The OS-unbound samples [Fig. 6(A), lanes 3 and 4] from

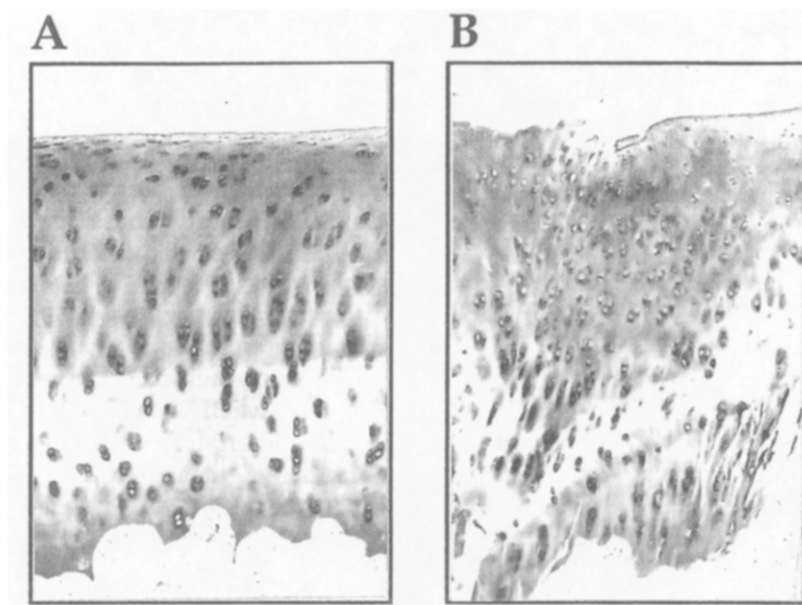


FIG. 2. Toluidine blue/fast green stained sections of osteochondral specimens from the dorsal radial facet of the third carpal bone. (A) Normal region and (B) region with a punctate cartilage depression. (Original magnification  $\times 10$ ).

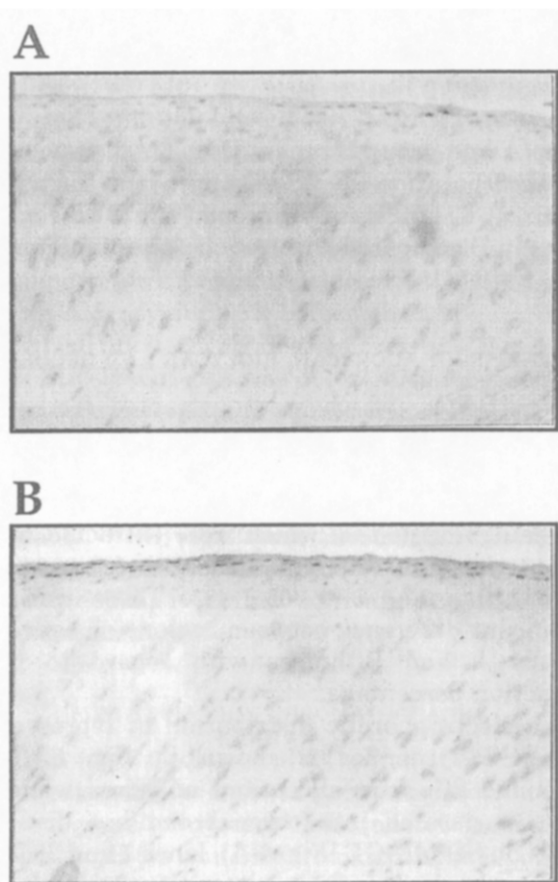


FIG. 3. Immunohistolocalization with monoclonal antibodies (A) 3B3(–) and (B) 7D4 in cartilage from the dorsal radial facet. The positive immunostaining of the surface chondrocytes was similar in all regions of both groups of horses. (Original magnification  $\times 10$ ).

all regions of all horses contained only the slowest migrating  $^{35}\text{S}$ -PG band. When the OS-bound material was digested with chondroitinase ABC before electrophoresis, the radioactivity in the intermediate and fast migrating bands was eliminated [Figure 6(A) lane 5] and a Coomassie stained core protein band ( $M_r \sim 45$  kDa) became apparent [Fig. 6(B), lane 2].

The radioactivity incorporated into the putative biglycan and decorin SDS-PAGE bands of all regions of group A and B horses were quantitated directly from the gels. The  $^{35}\text{SO}_4^{2-}$  incorporated into aggrecan was calculated by the addition of the radioactivity in the OS-unbound material and that in the slowest migrating SDS-PAGE band in the OS-bound sample. In all cartilage regions of both groups of horses the synthesis of aggrecan was significantly higher than decorin ( $P < 0.0001$ ) or biglycan ( $P < 0.0001$ ). Decorin synthesis was significantly higher than biglycan in the DRF and DIF regions of both groups of horses ( $P < 0.006$ ) but not the PC in either group A or B animals (Fig. 7). The synthesis of aggrecan, biglycan or decorin was not significantly different between the three regions of group A horses [Fig. 7(A), (B) and (C), respectively]. The synthesis of aggrecan was significantly lower ( $P = 0.049$ ) in the DRF of group B compared with group A horses [Fig. 7(A)]. The synthesis of decorin was significantly higher in the DRF of group B compared with group A horses [Fig. 7(B)]. Furthermore, in group B animals the synthesis of decorin was significantly higher in the

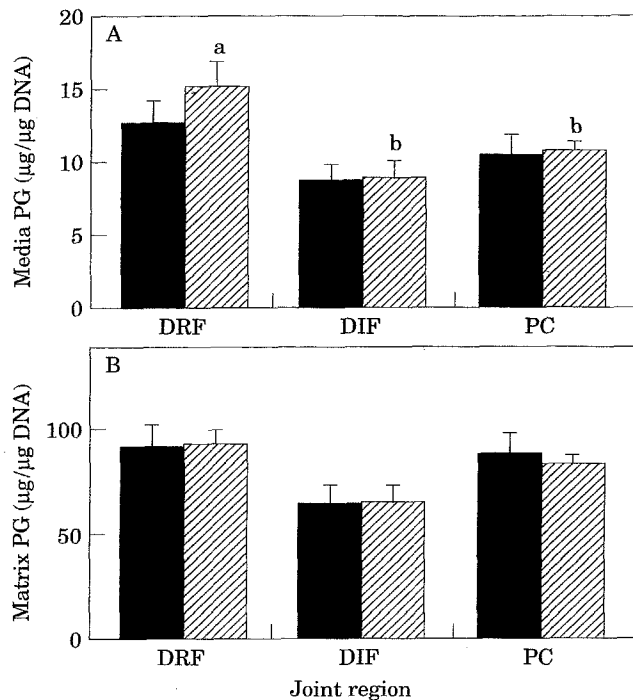


FIG. 4. Resident proteoglycans (DMB positive PGs) (A) released into the culture media and (B) retained in the cartilage matrix ( $\mu\text{g}/\mu\text{gDNA}$ ; mean + standard error). Abbreviations for joint regions are as described in Fig 1. (■) Group A; (▨) Group B.

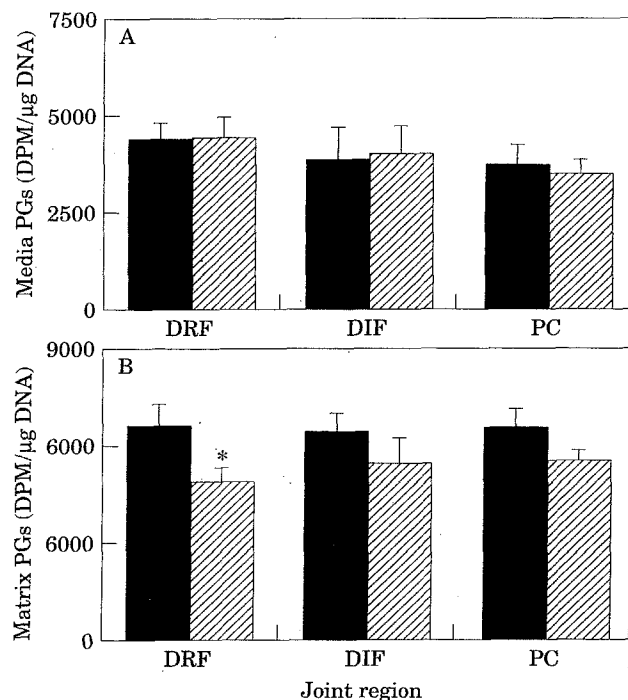


FIG. 5. Newly synthesized proteoglycans ( $^{35}\text{S}$ -PGs) (A) released into the culture media and (B) retained in the cartilage matrix (DPM/ $\mu\text{gDNA}$ ; mean + standard error). \*Significant difference between group A and B horses. (■) Group A; (▨) Group B.

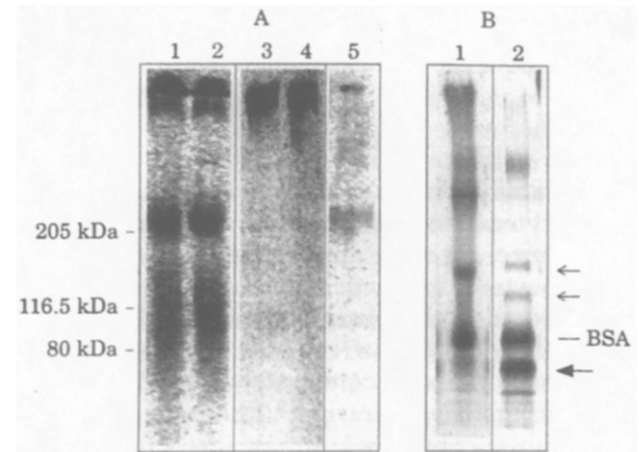


FIG. 6. Representative examples of the 4–12% SDS-PAGE separation of equine cartilage extracts from the dorsal radial facet (A) Phosphorimage of  $^{35}\text{S}$ -PGs: lane 1 = group A OS-bound, lane 2 = group B OS-bound, lane 3 = group A OS-unbound, lane 4 = group B OS-unbound and lane 5 = lane 1 digested with Chondroitinase ABC prior to electrophoresis. (B) Coomassie Brilliant Blue R250 stained gel, lane 1 = OS-bound and lane 2 = OS-bound digested with Chondroitinase ABC prior to electrophoresis. Open arrows indicate proteins present in the Chondroitinase ABC solution, solid arrow indicates 45 kDa DS-PG core protein band which became apparent after Chondroitinase ABC digestion and BSA = bovine serum albumin.

DRF than both the DIF and PC ( $P=0.009$  and  $0.0007$ , respectively). There was no difference between group A and B horses with regard to the synthesis of biglycan in any cartilage region [Fig. 7(C)].

#### SIZE EXCLUSION CHROMATOGRAPHY

There was no difference between the two groups of horses or between regions with regard to the size distribution of resident or  $^{35}\text{S}$ -PGs on Sephacryl S1000 chromatography (data not shown). The resident PGs in the culture media separated into two major pools, one which aggregated with HA and voided the column ( $K_{av}=0$ ) and the other which failed to aggregate and was included ( $K_{av}\sim 0.5$ ). In contrast, the majority of the  $^{35}\text{S}$ -PGs released into the media formed a broad included peak ( $K_{av} 0.75$ ) with a shoulder at  $K_{av}\sim 0.5$ . The resident and newly synthesized OS-unbound PGs eluted as a single broad peak when chromatographed under dissociative conditions. The newly synthesized PGs were somewhat larger than the resident molecules ( $K_{av} 0.35$  compared with  $0.4$ ). Under associative conditions the majority ( $72 \pm 0.9\%$  for group A and



74  $\pm$  1.7% for group B) of OS-unbound PGs aggregated with HA and voided the column.

### Discussion

This study has demonstrated that strenuous running exercise in horses can lead to a disturbance in the metabolism of PGs by chondrocytes in articular cartilage regions subjected to high contact stresses (DRF). These metabolic changes were not associated with widespread gross or histomorphological evidence of cartilage degeneration. Punctate cartilage depressions considered to be associated with subchondral bone collapse were observed with similar frequency in the radial facet of both groups

of horses (Fig. 2). Similar lesions have been previously reported in Standardbred and Thoroughbred horses as incomplete slab fractures or subchondral lucency of the third carpal bone [18, 29, 30]. It was suggested in these previous reports that these changes in the subchondral bone may predispose to carpal fractures, and were likely a result of focal mechanical stress in the radial facet of the third carpal bone. It is unclear whether these lesions were present prior to the commencement of the study as a result of previous training and racing, or represent abnormalities induced by both the current exercise protocols. Due to budget limitations, a non-exercised control group was not available for comparison with the two exercise protocols in the current study. Furthermore, acquisition of skeletally mature research horses which have never undergone any previous enforced exercise program is cost prohibitive because they must be purchased as yearlings and maintained for several years. The lack of sedentary controls in the present study limits the conclusions that may be drawn on PG metabolism in resting versus exercising horses. However, the comparison between the two different exercise protocols remains valid, and several important observations can be made.

Increased levels of PGs bearing 3B3(-) and 7D4 epitopes in the articular cartilage matrix has been observed with the onset of OA in other species including man [28, 31–34]. The increase in PGs reacting with 3B3(-) and 7D4 antibodies is associated with a hypermetabolic response of chondrocytes and has been used as a marker of early OA change [28, 31–34]. Increased 3B3(-) levels have also been induced in normal bovine cartilage that was cyclically loaded *in vitro*, suggesting a role for mechanical regulation of synthesis of this epitope [35]. While 3B7(-) and 7D4 epitopes were evident in the superficial cells of articular cartilage in the present study, there was no matrix staining in any samples and no difference between joint regions or exercise groups in the levels of these markers. It is not clear whether both the present exercise protocols had induced any increase in the level of expression of these epitopes compared with resting horses. Staining restricted to the superficial chondrocytes, as observed in the present study, has however been reported as normal in other species [28, 34]. Given that abnormalities in PG metabolism were only detected in the DRF region, this suggests that the level of expression of these epitopes observed in this study was not indicative of pathological change. Furthermore the PC and DIF regions are subjected to less mechanical loading than the DRF

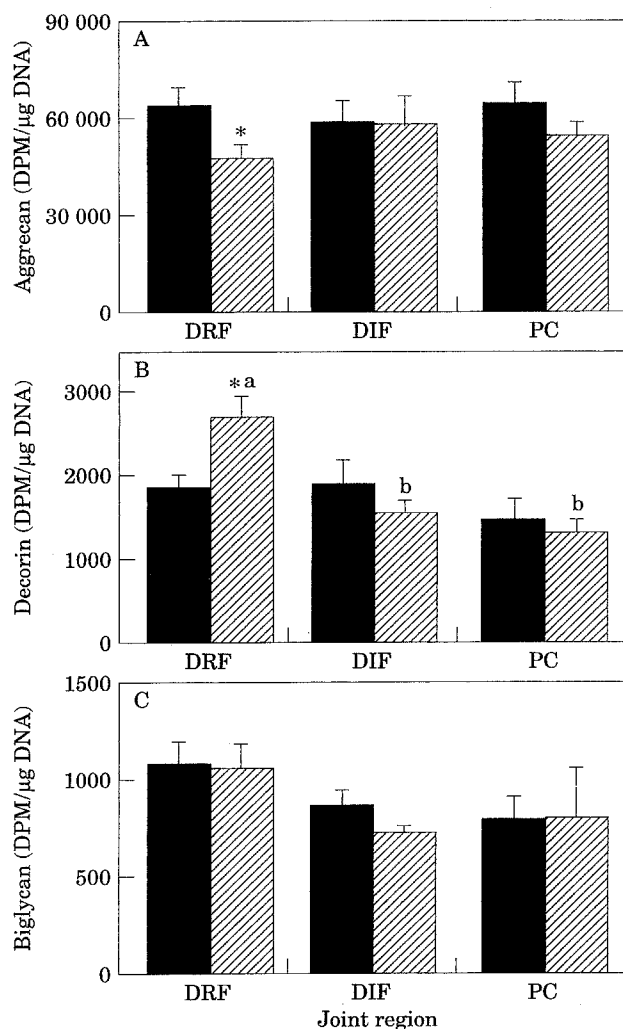


FIG. 7. Incorporation of  $^{35}\text{SO}_4^{2-}$  into (A) aggrecan, (B) decorin and (C) biglycan (DPM/ $\mu\text{gDNA}$ ; mean  $\pm$  standard error) in cartilage from the different joint regions of Group A and B horses. \*Significant difference between group A and B horses ( $P < 0.05$ ). a,b = Columns with different letters have significantly different values from each other ( $P < 0.01$ ). (■) Group A; (▨) Group B.



and yet these areas showed similar localization with 3B3(-) and 7D4 to the more highly loaded DRF in both groups of horses. We would therefore suggest that the degree of positive immunostaining observed with both antibodies in the present study represents a normal distribution in adult horses undergoing exercise.

Despite the lack of OA change in the articular cartilage, as detected by histology and immunohistology, abnormalities in PG metabolism were observed in the strenuously exercised group of horses. In high weight-bearing regions (DRF) of group B horses there was a significant decrease in total PG synthesis (measured as  $^{35}\text{SO}_4^{2-}$  incorporation) when compared with the moderately exercised group. This reduction in  $^{35}\text{SO}_4^{2-}$  incorporation was largely due to a decrease in aggrecan synthesis. In group B but not group A animals, the DRF articular cartilage released significantly more PGs into the culture media than other joint regions. This finding was associated with a trend towards increased release of PGs from this region in the strenuously compared with moderately exercised animals. The size exclusion chromatograms of PGs released into the culture media in both groups indicated that the released PGs were consistent in size with partially degraded aggrecan monomers as has been described by others [26, 36]. Proteolytic cleavage of the aggrecan core protein in the interglobular domain (IGD) releases a large GAG rich fragment which is no longer capable of aggregating with HA and diffuses from the tissue [37, 38]. Numerous proteinases of the metalloproteinase class have been shown to cleave aggrecan in the IGD [39, 40]. However, it has become apparent from N-terminal amino acid analysis of the aggrecan cleavage products in other species that another, as yet unidentified enzyme (termed 'aggrecanase'), may be primarily responsible for aggrecan turnover particularly under pathologic conditions [41, 42]. Whether 'aggrecanase' is involved in the catabolism of cartilage aggrecan in horses is unknown. Further analysis of the degradation products released into the culture media from explants in the present study has not been attempted to date, but would clearly be of considerable interest. An increase in release of PG fragments into the culture media in strenuously exercised horses would be consistent with the elevated synovial fluid GAG concentration in exercised horses previously reported by others [43, 44].

Despite the reduced incorporation of newly synthesized aggrecan into the cartilage matrix and the increased degradation and release of resident

aggrecan molecules by the DRF articular cartilage in group B compared with group A horses, the PG content of cartilage from the two groups of animals was not different at the time of sacrifice. The explanation for this apparent contradiction is not clear. The above described alterations in PG metabolism may not as yet be sufficiently great to reflect measurable differences in the overall PG content of the cartilage. Alternatively the strenuous exercise program may have initially induced an increase in the PG content of the cartilage compared with moderate exercise. Consistent with this hypothesis, low to moderate intensity training over 6 weeks has been shown to increase PG synthesis in articular cartilage from all regions of the equine third carpal bone [45]. The decrease in PG synthesis and increase in PG catabolism observed in the present study may therefore only have become evident in the latter stages of the strenuous exercise program. The single time at which cartilage was sampled in the present study may therefore have coincided with a point at which the PG content of the strenuously exercised group had returned to a similar level as that in the moderate exercise group. It may be expected that the continued reduction in synthesis of aggrecan in the DRF of strenuously exercised animals would ultimately lead to a decline in the cartilage matrix concentration of PG in this region. Such a change would have important implications on the load dissipating ability of the tissue. Loading, even within the physiological range, of aggrecan depleted cartilage has been shown in other species to result in mechanical disruption of the tissue [46]. It is noteworthy that the size and the ability of the newly synthesized aggrecan monomers to aggregate with HA was not appreciably different between the two groups of horses in the present study. Thus, while the synthesis of aggrecan was decreased in the DRF of group B horses, there was no apparent functional abnormality in the PGs that were produced.

The separation of OS-bound PGs by SDS-PAGE described in the present study were identical to those reported using the same methodology with ovine articular cartilage PGs [15]. In this previous report the identity of biglycan and decorin (SDS-PAGE bands 2 and 3, respectively, in the OS-bound PGs) were confirmed with the use of specific antibodies. In the present study Western blotting with a variety of antibodies to biglycan and decorin of other species was attempted, but the equine PGs failed to cross react (data not shown). Nevertheless, the hydrophobicity, SDS-PAGE distribution and core protein size of the equine OS-bound small PGs was consistent with the

assignment of the intermediately migrating band as biglycan and the most mobile band as decorin. Additionally chondroitinase ABC digestion of the samples prior to electrophoresis indicated that the radioactivity in these bands was associated with chondroitin/dermatan sulfate rather than keratan sulfate, indicating that contamination of these bands with aggrecan breakdown products had not occurred.

An increase in decorin synthesis was observed in the DRF cartilage of group B compared with group A horses. This was consistent with previous reports on the effect of mechanical loading of articular cartilage *in vitro*. A threefold increase in total DS-PG synthesis was observed in articular cartilage following 7 days of cyclic *in-vitro* loading by Korver *et al.*, (1992) [47]. More recently it has been found that cyclic mechanical loading of both adult and immature bovine articular cartilage increased the synthesis of decorin but not biglycan [48, 49]. Thus the increase in decorin synthesis in the DRF of group B horses may reflect the increase in mechanical loading experienced by this joint region. The role of decorin in the cartilage extracellular matrix has not been fully elucidated, however *in-vitro* studies of collagen fibrillogenesis have shown that decorin addition results in the production of more uniform and thin collagen fibrils [12]. Furthermore, an increase in decorin concentration has been associated with the loosening of the collagen fibrillar network which develops in the peri-parturient uterine cervix of both rats and women [50, 51]. A change in the decorin:collagen ratio as a result of the observed increase in decorin synthesis in the DRF articular cartilage of group B horses, may alter the arrangement of the collagen fibrillar network in this highly stressed region. This would be consistent with the loosening of the collagen network and softening of the cartilage observed in strenuously exercised dogs [16, 17], and may predispose the cartilage to subsequent mechanical disruption. In this regard, we have previously observed an increase in decorin synthesis in the articular cartilage of sheep in the early stages of experimentally induced OA [15].

It is noteworthy that the metabolic changes observed in the present study were apparent some 16 weeks after the cessation of exercise. We consider therefore, that these abnormalities in PG biosynthesis by chondrocytes subjected to high mechanical stresses, identifies a change in their phenotypic expression. The observed alteration in chondrocyte phenotype may be irreversible and could represent a predisposing factor for cartilage degeneration and OA at a later stage. Further

study of the effects of different training/exercise regimens on the metabolism of articular cartilage in horses may provide valuable information on the pathogenesis of OA in this species. It is only with a thorough understanding of the molecular mechanisms involved in cartilage degradation in both normal and pathological equine joints, that rational approaches to the treatment and ultimately prevention of OA in athletic horses may be devised.

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